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Original article

Combined effects of antioxidant vitamin and *NOS3* genetic polymorphisms on breast cancer risk in women

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SUMMARY

Background & aims: It is becoming increasingly clear that there is wide heterogeneity in genetic predisposition to breast cancer and that breast cancer risk is determined by interactive effect between genetic and environmental factors.

Methods: We investigated the combined effects of antioxidant vitamin intake and *NOS3* genetic polymorphisms on breast cancer risk in a Korean population (Seoul Breast Cancer Study). Histologically confirmed breast cancer cases ($n = 512$) and age, menopause status-matched controls ($n = 512$) with no present or previous history of cancer were recruited from several teaching hospitals in Seoul during 2001–2003. Two genetic polymorphisms of *NOS3* (298G > T and −786 T > C) were assessed by single base extension assays.

Results: No overall association between the individual *NOS3* genotypes or diplotypes and breast cancer risk was found, although the difference between cases and controls in the frequency of the *NOS3* 894 G > T polymorphism showed borderline significance (OR = 0.74, 95% CI = 0.52–1.06). There was no significant difference in energy intake or the intake of antioxidant vitamins between cases and controls, with the exception of vitamin E (OR = 0.49 lowest vs. highest quartile, $P_{trend} < 0.01$). On the other hand, our results suggest that antioxidant vitamin intake may modify the effects of the *NOS3* −786 T > C or 894 G > T genetic polymorphisms on breast cancer risk. Although a multiplicative interaction was not observed, the protective effect of β-carotene intake on breast cancer risk was observed predominantly in individuals with the TG:TG diplotype of NOS3 (OR = 0.68) but not observed with others diplotype. An inverse association between vitamin E intake and breast cancer risk was observed for individuals with the NOS3 786 TC + TT genotype and the NOS3 894 GG genotype. In addition, folic acid had a protective effect in the NOS3 786 TT and NOS3 894 GT + TT genotype.

Conclusion: Our results suggest that intake of antioxidant vitamins might modify the association between genetic polymorphisms of *NOS3* and breast cancer risk.

1. Introduction

The body of literature on the combined effects of genetic polymorphisms and various dietary factors on breast cancer risk is steadily increasing.[1] Heterogeneity in genetic predisposition related to oxidative stress, carcinogen-metabolizing genes, and the loss of balance between pro- and antioxidant processes may be important in the etiology of breast cancer.[2] The balance of endogenous oxidant and antioxidants, including dietary antioxidant vitamins, is likely affected by variations in genes involved in the generation and removal of oxidative species.[3–6]

Nitric oxide synthase (NOS3) can be upregulated by increased reactive oxygen species (ROS) levels[7,8]; this enzyme catalyzes the production of low (nanomolar) levels of nitric oxide (NO). NO is a multifunctional, short-lived molecule that can have both carcinogenic and anticancer effects, depending upon a number of factors.[9–11] At low levels, NO is considered to be cytoprotective and can act as an antioxidant by scavenging for ROS.[12] However, NOS3 expression has been detected in breast cancer tumors[13–15] and is positively associated with estrogen and progesterone receptor status.[14,15] NO may

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protect against ROS by interacting with metals to prevent the formation of oxygen species, scavenging oxidants, and/or preventing the degradation of metalloproteins and lipid peroxidation.[16] NO is a highly reactive free radical that plays a role inrowth, progression, or tumor metastasis[17] depending on its concentration, the local microenvironment, and when it is produced.[18] Among the many polymorphisms identified in NOS3 gene, two functional SNPs have been investigated the promoter (−786 T > C: rs2070744)[19] and the missense mutation (894G > T, Glu298Asp: rs1799983).[20] We suggested that genetic polymorphisms in NOS3 may modify risk for invasive breast cancer with lymph node involvement in Korean women through our previous study.[21] Here, we examined whether intake of the antioxidant vitamins beta-carotene, vitamin E, vitamin C, and folic acid modified the associations between NOS3 genetic polymorphisms and breast cancer risk.

2. Material and method

2.1. Subjects

This study based on the Korean Breast Cancer Study (KoBCS) which described more detail information in our previous study.[21] Cases for this study were breast cancer patients admitted to two teaching hospitals (Seoul National University Hospital and Asan Medical Center) located in Seoul, Korea. The control subjects were comprised of non-cancer patients admitted to the same hospitals and healthy women participating in a health check-up program performed in another hospital, Ewha University Hospital, also located in Seoul. Finally, 1026 cases and 1484 controls were recruited during 2001–2003.

After exclusion of those with previous history of cancer, hysterectomy and oophorectomy, and those for whom DNA samples were unavailable, histologically confirmed breast cancer cases ($n = 512$) and age, menopause status-matched controls ($n = 512$) with dietary information were included in this study finally. The study design was approved by the Committee on Human Research of Seoul National University Hospital, and the subjects provided informed consent prior to participation in the study.

Information on demographic characteristics, education, marital status, family history of breast cancer in first- and second-degree relatives, reproductive and menstrual factors, life-style habits, were collected by trained interviewers using a structured questionnaire.

2.2. Genotyping

DNA was isolated using Qiagen Blood Kit (Qiagen, Chatsworth, CA, USA), from blood drawn into 10-ml heparinized tubes and stored at −20 °C until use. Genotypes of the promoter region (−786 T > C: rs2070744) and exon 7 (894G > T, Glu298Asp: rs1799983) polymorphisms in NOS3 were determined by the SNP-ITTM assay using the SNP stream 25 KTM System (Orchid Biosciences, NJ, USA). Polymerase chain reaction (PCR) primers and SNP-ITTM primers used in the assays are listed in our previous report.[22]

Briefly, the genomic DNA region spanning the polymorphic site was PCR-amplified using one phosphothiolated primer and one regular PCR primer. The amplified PCR products were then digested with T7 exonuclease. The 5′-phosphothioates protected one strand of the PCR product from T7 exonuclease digestion, resulting in the generation of a single-stranded PCR template. The single-stranded PCR template was overlaid onto a 384-well plate that contained a covalently attached SNP-ITTM primer extension primer designed to hybridize immediately adjacent to the polymorphic site. The SNP-ITTM primer was extended for a single base with DNA polymerase and a mixture of appropriate dye-labeled acyclic nucleoside triphosphates, complementary to the polymorphic nucleotide that was labeled with either FITC or biotin. Incorporated nucleotide was determined by serial colorimetric reactions with anti-FITCAP and streptavidin-HRP, respectively. Resultant yellow and/or blue color development was analyzed using an ELISA reader and the final genotype calls were made using a QCReviewTM program (Orchid Biosciences, NJ, USA).

The reliability was tested for four samples with ten repeats randomly placed in the 384-well plates. Out of 80 quality control reactions (40 samples for 2 loci), the concordance rate was 99.8% (1 reaction result was indeterminate). The successful genotyping call rate per sample is 1.86% and the call rate for each SNPs are 1.17% and 2.54% for −786 T > C and 894G > T, respectively. All loci of the tested genotypes of NOS3 were in Hardy–Weinberg equilibrium.

2.3. Measurement of nutrient intake

A validated food-frequency questionnaire (FFQ) was used to assess usual dietary intake at the survey. The FFQ was consisted of 56 dish and food items, which included a general Korean meal items and their cooking methods. The detail information of FFQ was described in our previous study.[23] During the in-person interviews, each participant was first asked how often, on average, during the past 12 months she had consumed a specific food or food group (possible responses were more than 3 times daily, 1–2 times daily, 5–6 times weekly, 3–4 times weekly, 1–2 times weekly, 2–3 times monthly, 1 time monthly, or never), followed by a question on the amount consumed with one serving size per unit of time. For seasonal food, such as fruits, participants were asked to describe their consumption during the season(s). Energy and nutrient including antioxidant intake were calculated by multiplying the amount of food consumed by the nutrient content per gram of the food, as obtained from the Korean Food Composition Tables.[24] The FFQ was validated against the average of multiple 24-h recalls.[23] Correlation coefficients between the FFQ and the 24-h dietary recall average were 0.74–0.88 for macronutrients and 0.43–0.85 for micronutrients.

2.4. Statistics

Risks were estimated as odds ratios (ORs) and 95% confidence intervals (CIs), by unconditional logistic regression, adjusting for age (continuous), body mass index (continuous), family history of breast cancer (yes/no), age of first full-term pregnancy (<25, 25–29, and ≥30 years old and nulliparous), and total energy intake (quartile).

After excluding the missing genotype data, individual haplotypes for NOS3 −786 T > C and 894G > T were estimated by the Bayesian method (PHASE ver. 2.0.2) (http://www.stat.washington.edu/stephens/software.html)[25] and Linkage disequilibrium (LD) was estimated as D′ from the estimated haplotype data. Estimated haplotypes with low accuracy (<90%) were excluded from the analysis. In diplotype (combination of haplotype) analysis, we categorized them into three groups; the diplotype consisting of the most common haplotypes (haplotype T-G) was used as the reference, the diplotype consisting the haplotype T-G and other haplotype, and the diplotypes containing all other haplotypes. We used omnibus test to assess differences in overall haplotype frequency profiles between cases and controls.[26]

Stratified analyses were performed to investigate any interaction or combined effect between the NOS3 genotype and selected antioxidant vitamins. The categories of the antioxidants intake were divided two as low and high using median. Tests for interaction were performed by comparing the model with and without interaction terms using a likelihood ratio test. All statistical tests